Detection of Smallpox Virus DNA by LightCycler PCR

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A 300-bp plasmid fragment of the hemagglutinin gene was used as target DNA to develop a rapid real-time LightCycler (Roche Applied Science, Indianapolis, Ind.) PCR assay for laboratory detection of smallpox virus. PCR primers and probes were designed specifically for detection of smallpox virus DNA, but all viruses of the genus *Orthopoxvirus* tested could be detected by use of the hemagglutinin gene target sequence. Base pair mismatches in the 204-bp amplicon allowed discrimination of cowpox virus (melting temperature [T_m], 56.40°C), monkeypox virus (T_m, 56.24°C), and vaccinia virus (T_m, 56.72°C), including the Dryvax vaccine strain, from smallpox virus (T_m, 62.45°C) by melting curve analysis. The analytical sensitivity was 5 to 10 copies of target DNA per sample. The assay was specific for members of the genus *Orthopoxvirus*; the DNAs of herpes simplex virus and varicella-zoster virus were not detected by the smallpox virus LightCycler PCR.

Smallpox virus is a major threat as an agent of bioterrorism. Clinical infection is characterized by an incubation period of 12 to 14 days (range, 7 to 17 days), followed by the onset of high fever, malaise, and prostration, often with severe headache, nausea, and vomiting (6). A rash that progresses from the papular to vesicular stages and eventually to the pustular stage generally appears by the third day of illness and initially appears in the mouth on the oral mucosa, on the face, and on the head and then spreads to the limbs and trunk of the body. Smallpox patients can transmit the virus during the first week, when the lesions in the oral mucosa ulcerate and seed the saliva with smallpox virus (6, 21, 23, 27, 28). The disease causes severe morbidity and can lead to a mortality rate of 30% among the victims.

The family *Poxviridae* and the subfamily *Chordopoxvirinae* (poxviruses of vertebrates) contain eight genera; importantly, smallpox virus and the closely related cowpox virus, vaccinia virus, and monkeypox virus (the vaccine strain used for immunization against smallpox) can infect humans and are classified in the single genus *Orthopoxvirus*. These DNA viruses are large brick-shaped particles that vary in size (length, 220 to 450 nm; width, 140 to 260 nm); the genome of smallpox virus consists of 186 kb of linear double-stranded DNA (19).

Two molecular amplification tests have previously been described for the detection and discrimination of viruses within the genus *Orthopoxvirus*; however, labor-intensive conventional PCR with consensus or sequence-specific primers and subsequent digestion of amplicons with several restriction endonucleases followed by gel electrophoresis were required to identify the specific viruses within this group (20, 25). Recently, reference strains of smallpox virus and scabs from patients (Russian archived specimens) with this infection were detected in a 6-h test by a homemade PCR amplification and detection system based on oligonucleotide microchip technology (17).

We describe a rapid (3-h [for nucleic acid extraction and amplification]) real-time PCR (LightCycler; Roche Applied Science, Indianapolis, Ind.) that detects smallpox virus and that differentiates the DNA of this agent from the DNAs of other viruses classified within the genus *Orthopoxvirus*.

MATERIALS AND METHODS

Virus strains. Cowpox virus (vr-302), monkeypox virus (vr-267), and vaccinia virus (vr-117) were obtained from the American Type Culture Collection, Manassas, Va. Vaccinia virus (Dryvax vaccine; Wyeth Laboratories, Inc., Marietta, Pa.) was obtained from the Centers for Disease Control and Prevention (CDC), Atlanta, Ga. *Orthopoxvirus* strains were propagated in MRC-5 cells. A 300-bp plasmid insert of a portion of the hemagglutinin (HA) gene was used as the smallpox virus target.

Nucleic acid extraction. Nucleic acids were extracted with a Total Nucleic Acid Isolation kit (catalog no. 2236931; Roche Applied Science Indianapolis, Ind.) with an automated MagNA Pure extraction instrument, as described previously (8).

LightCycler PCR. The LightCycler instrument (Roche Applied Science) was used to amplify (in approximately 45 min) target DNA and to monitor the development of the PCR product after each cycle (denaturation, annealing, and extension). To monitor the PCR product, dual fluorescence resonance energy transfer (FRET) probes were used. PCR primers were designed to amplify a segment of the smallpox virus HA gene, and the FRET probes were designed to anneal to an internal nucleotide sequence of the resultant 204-bp product. The PCR master mixture, including uracil-*N*-glycosylase, was optimized for detection of the target DNA in the HA gene of smallpox virus by adding 2% dimethyl sulfoxide, 4 mM $MgCl_2$, and 0.7 μM primers. The cycling protocol was 37°C for 5 min and 95°C for 3 min for 1 cycle, followed by 95°C (no holding time), 12 s of annealing at 55°C, and 12 s of primer extension at 72°C for 45 cycles.

Melting curve for *Orthopox* genotype analysis. Differentiation of smallpox virus, cowpox virus, monkeypox virus, and vaccinia virus was obtained by detection of nucleotide sequence mismatches with the FRET probes and by use of the melting curve feature of the LightCycler PCR software. Starting at 45°C, the temperature in the thermal chamber was held constant for 1 min and was then slowly raised to 80°C; the fluorescence was measured at frequent intervals. Sequence differences between the PCR product and the hybridization probes resulted in shifts in the melting temperatures (T_ms) unique for smallpox virus, cowpox virus, monkeypox virus, and vaccinia virus.

Plasmid preparation. Positive controls for members of the genus *Orthopoxvirus* were generated by producing a clone with the pCR 2.1 TOPO TA (Invitrogen Corp., Carlsbad, Calif.) cloning kit. Each of the PCR amplicons of smallpox

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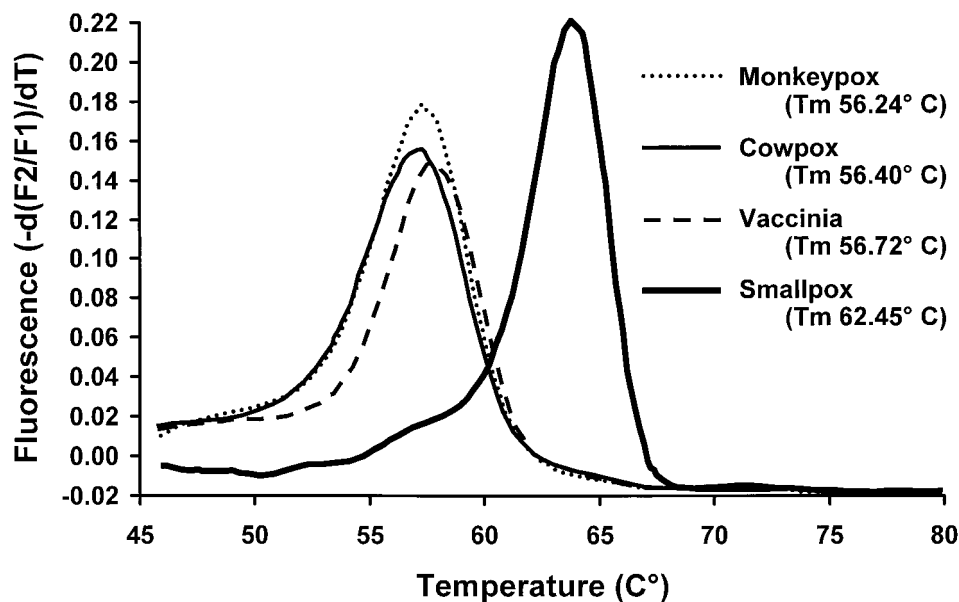
FIG. 1. Melting curve analysis of orthopoxviruses by LightCycler PCR.

virus, cowpox virus, monkeypox virus, and vaccinia virus were individually inserted into separate plasmid vectors; the recombinant vectors were transformed in chemically competent *Escherichia coli* cells. The correct recombinant plasmid was confirmed and purified with a Wizard MiniPrep (Promega Corp., Madison, Wis.) DNA Cleanup kit. The stock concentrations of the controls (in genomic equivalents) were determined.

RESULTS

All viruses of the genus *Orthopoxvirus* tested (cowpox virus, monkeypox virus, and three strains of vaccinia virus [two from the American Type Culture Collection and the Dryvax vaccine strain]) produced amplified DNA products (204 bp) detectable by FRET analysis with primers and probes whose sequences were homologous to a portion of the sequence of smallpox virus DNA within the HA gene. The DNA of each orthopoxvirus (cowpox virus, monkeypox virus, vaccinia virus) differed from smallpox virus DNA by base pair mismatches that allowed distinct recognition and discrimination of their target DNAs by melting curve analysis. Since the assay was designed to be specific (homologous) for the DNA of smallpox virus, the target DNA, the T_m of the PCR product from smallpox virus DNA was the highest of those tested (T_m, 62.45°C); the 2-bp (vaccinia virus), 3-bp (monkeypox virus), or 4-bp (cowpox virus) differences in the nucleotide sequences of the other orthopoxviruses produced melting curve shifts specific for cowpox virus (T_m, 56.40°C), monkeypox virus (T_m, 56.24°C), and vaccinia virus (T_m, 56.72°C) (Fig. 1). The assay had an analytical sensitivity of 5 to 10 copies of genomic DNA per reaction mixture for all orthopoxviruses tested.

The smallpox virus PCR assay was specific for viruses within the genus *Orthopoxvirus*. Similarly, smallpox virus target DNA was not amplified by LightCycler PCR assays for herpes simplex virus (HSV) or varicella-zoster virus (VZV), which we routinely perform in our laboratory for the diagnosis of infections caused by those viruses (7, 9, 10, 29).

DISCUSSION

The combination of the characteristics of smallpox virus (stable virion, person-to-person transmission, high rates of morbidity and mortality [30%]) and the existence of a crowded and mobile population of susceptible hosts worldwide portend the imminent importance of this virus as an agent of bioterrorism (2, 18). The availability, cultivation, and stockpiling of smallpox virus for biological warfare or bioterrorism by several countries have been recognized (1, 4, 11, 16). Extensive preparations and resources have been focused on infection control efforts to respond to a bioterrorism event involving smallpox virus; these efforts are described on the CDC website (www.cdc.gov).

Activation of bioterrorism response plans depends on the immediate clinical suspicion and recognition of the disease; this step must be followed by the rapid and accurate laboratory diagnosis of the smallpox virus infection (12). Herpesvirus (HSV and VZV) infections, erythema multiforme, allergic dermatitis, and infections with other orthopoxviruses such as monkeypox virus must be considered in the differential clinical diagnosis of smallpox virus infection (5, 14). Routine vaccination for prevention of smallpox virus infection was discontinued in the United States in 1972. Furthermore, in 1980, the World Health Assembly certified that the world was free of naturally occurring smallpox. Because of partial immunity related to previous vaccination in some individuals, patients with smallpox virus infection may not have typical clinical presentations in terms of their symptomatologies and the distributions of dermal lesions that were described in unvaccinated patients with smallpox during outbreaks of this disease more than 30 years ago. Particularly in these situations, an immediate and rapid laboratory diagnosis can facilitate the public health response, including appropriate communications to the

public regarding the need for any control measures (15, 18, 21, 26, 31).

Recently, a method (MAGIChip; a 6-h procedure) was described that used hybridization of a fluorescence-labeled amplified DNA specimen to reference *Orthopoxvirus* oligonucleotide DNA probes which were immobilized on a microchip (17). The assay was specific and correctly distinguished 16 reference strains from the Russian collection of smallpox viruses. In addition, the assay detected this virus in scab specimens tested in a biosafety level 4 laboratory of the Russian State Research Center of Virology and Biotechnology "Vector" and specifically identified it apart from other members of the genus *Orthopoxvirus*. Importantly, for implementation of this test procedure in other laboratories throughout the world, considerable technical expertise in the preparation of specific oligonucleotide probes will be required; in addition, access to the DNA of the target gene of each virus is required for validation of the test in every laboratory. However, aside from the hybridization and optimization of the molecular amplification protocol, the procedure does not require any sophisticated equipment; only a portable fluorescence reader is required for the detection of patterns.

The Division of Clinical Microbiology of the Mayo Clinic has had 3 years of experience in the design of PCR primers and probes for the development, optimization, and implementation of rapid real-time PCR with the LightCycler instrument. Tests for the laboratory diagnosis of infections caused by HSV (genital and dermal specimens), VZV (dermal specimens), and cytomegalovirus (urine specimens) have been implemented since May 2000 and have replaced shell vial cell culture assays for the routine detection of these viruses (29). As first-line medical sentinel personnel, physicians will require rapid and specific assays (commercially available assays with analyte-specific reagents) for the rapid detection of smallpox virus infections; in addition, they will also require laboratory resources for the immediate diagnosis of HSV and VZV infections in patients who present with atypical clinical features that may mimic those produced by smallpox virus. The rapid cycling temperatures achieved by alternating heated air with air of ambient temperature in a closed system that continuously monitors samples for amplicon development with a fluorometer within the LightCycler instrument allow this PCR to be incorporated into the clinical laboratory. The PCR assay was analytically sensitive, and its performance characteristics were similar to those of other assays for viruses formatted for the LightCycler instrument, in that 5 to 10 copies of smallpox virus DNA could be detected. Most importantly, the DNA did not react with herpesvirus (HSV, VZV) target nucleic acids or with cellular nucleic acids extracted from uninfected MRC-5 cells.

PCR with the LightCycler instrument is a rapid and accurate test for the detection of smallpox virus DNA and can provide first-line support for the physician who suspects on clinical grounds that a patient has smallpox virus infection. At present, biosafety recommendations for the processing of lesion fluid or crust specimens, respiratory secretions, or tissue for laboratory diagnosis of smallpox virus infection require the use of a level D (biosafety level 4) containment facility and expertise in the diagnosis of infections caused by rare or highly dangerous agents. Techniques such as immunofluorescence, electron microscopy, and more recent versions of PCR have been used for the conventional laboratory diagnosis of infections caused by rare or highly dangerous agents (3, 13, 17, 20, 22, 24, 25, 30). In the United States, only CDC and the U.S. Army Medical Research Institute of Infectious Diseases maintain level D laboratories that can provide direct laboratory support for the detection of infectious disease outbreaks. The requirement that only level D laboratories can test specimens for smallpox virus presents obstacles for the rapid, efficient, and safe transport of specimens. Any delay in the transport process has important implications in terms of the need for the use of quarantine measures until definitive results can be obtained by a level D laboratory (21). In another study by our group (M. J. Espy, J. R. Uhl, L. M. Sloan, J. E. Rosenblatt, F. R. Cockerill III, and T. F. Smith, Abstr. 102nd Gen. Meet. Am Soc. Microbiol. 2002, abstr. 2277, 2002), we have demonstrated that the infectivity of the closely related vaccine strain (vaccinia virus) of smallpox virus, in addition to the infectivities of HSV and VZV, are readily destroyed by autoclaving of the specimen. This procedure eliminates the subsequent threat of laboratory-acquired smallpox virus infection but does not affect the viral DNA that is the target of the PCR with the LightCycler instrument. Alternatively, the use of PCR assays for smallpox virus, HSV, and VZV DNAs with the LightCycler instrument, in combination with standard sterilization procedures with an autoclave (which is commonly available in almost every medical facility) for inactivation of smallpox virus in patient specimens, permits the laboratory diagnosis of smallpox virus infections caused by possible bioterrorism events by qualified laboratories at the local level.

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